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(54) Title: SERPIN VARIANTS

(57) Abstract

Modified antithrombin III variants are developed which contain amino acid substitutions in the region comprising amino acids 384-396. In this region one or more amino acids are replaced by the corresponding amino acid(s) grouped around the factor Xa cleavage site in factor II related to the formation of meizothrombin. These amino acid substitutions result in altered heparin-dependent inhibitory effect of the modified antithrombin III vis-à-vis the factors IIa and Xa, respectively.

* See back of page

Serpin variants

The invention is concerned with a modified antithrombin III (ATIII) polypeptide, a polynucleotide sequence coding therefore, a recombinant vector containing such a polynucleotide sequence, a method for the preparation of said ATIII polypeptide, as well as a pharmaceutical product containing the modified ATIII polypeptide.

Blood clotting is a dynamic process wherein a great number of enzymes and factors cooperate ultimately leading to a conversion of the fibrinogen circulating in the blood into a network of fibrin aggregates.

These blood clotting enzymes are activated consecutively and under each others action. The conversion of fibrinogen into fibrin takes place under direct action of thrombin (factor IIa), which in turn is formed from prothrombin (factor II), under the action of an enzyme complex containing among others factor Xa. Both factor IIa and factor Xa belong to the enzyme class serine endopeptidases. The activities of these factors leading to the conversion of fibrinogen into fibrin in turn are balanced by the so-called serine proteinase inhibitors (serpins). One of the most well-known serpins is antithrombin III, a protein able to bind to both factor IIa and factor Xa and then form a stabile enzyme-pseudosubstrate complex with these respective factors. The formation of such a complex is stimulated by heparin, and leads to reduction of the amounts of factor IIa and factor Xa available for fibrinogen conversion.

This stimulation is the basis of the therapeutic use of heparin.

However, a disadvantage of the use of heparin is that both factors IIa and Xa are inactivated to an about equal extent. This results in a relatively small therapeutic margin for heparin. Inhibition of one of the coagulation factors would improve this therapeutic margin. Therefore the present invention is concerned with modified ATIII polypeptides that in a heparin-dependent way can specifically inactivate either of the two enzymes.

A modified ATIII polypeptide according to the invention is characterized by an amino acid sequence which differs from the sequence of natural mature ATIII protein (represented in figure 1 as cDNA sequence) to such an extent that at least one of the amino acids in the region comprising amino acids no. 384-396 is replaced by the corresponding amino acid(s) grouped around the factor Xa cleavage-site in prothrombin (factor II) related to the formation of meizothrombin. These corresponding amino acids of factor II are represented in figure 2, together with the indicated polypeptide region of ATIII. Advantageously, one or more of the amino acids no. 384-389, 394, and 396 of ATIII are substituted according to the following scheme:

Ala^{384} → Glu
Ser^{385} → Leu
Thr^{386} → Leu
Ala^{387} → Glu
Val^{388} → Ser
Val^{389} → Tyr
Ala^{391} → Asp
Ser^{394} → Ile
Asn^{396} → Glu

Modified ATIII polypeptides according to the invention are exemplified in figure 2 as mutants 1-10.

The abovementioned substitutions result in remarkable shifts in the biochemical characteristics of the polypeptide.

In the presence of heparin the mature unmodified ATIII shows irreversible inhibition of the activities of both factors IIa and Xa. In contrast, polypeptides according to the invention wherein at least one of the amino acids no. 384-389 and 391 is replaced belong to the so-called type IIa inhibitors: in the presence of heparin they show a conserved irreversible inhibition of factor IIa, whereas their irreversible inhibition of factor Xa is lost (figure 3).

On the other hand, polypeptides wherein at least one of the amino acids no.391, 394 and 396 is replaced belong to the so-called type Xa inhibitors: in the presence of heparin they show a conserved irreversible inhibition of factor Xa, whereas their irreversible inhibition of factor IIa is lost (figures 3 and 4). In particular, a single amino acid substitution at position 394 can be solely responsible for the conservation of irreversible inhibition of factor Xa and the loss of irreversible inhibition of factor IIa. Modified ATIII polypeptides according to the invention are exemplified in figure 5 as mutants 11-18. The size and/or hydrofilicity of the amino acid at position 394 determines the heparin-dependent inhibition profile of the ATIII polypeptides (figure 6).

Hence, it has been found that substitution in ATIII of the amino acids in the region 384-396 offers the possibility to specifically modulate the heparin-dependent activity of the polypeptide.

The ATIII variants according to the present invention can be applied therapeutically to influence blood clotting disorders, more specifically e.g. deep venous thrombosis, disseminated intravascular coagulation, and septic shock by either i.v. or s.c. route.

The polypeptides according to the invention can be prepared with the aid of recombinant DNA technology. Herein a polynucleotide, coding for such a polypeptide is brought to expression. Such a polynucleotide can be prepared e.g. by modifying a polynucleotide sequence coding for ATIII by nucleotide substitutions in such a way that the resulting polynucleotide codes for a polypeptide according to the present invention. Another possibility is to synthesize a polynucleotide coding for the desired polypeptide, using known polynucleotide synthesis techniques. It goes without saying that both techniques mentioned also can be combined, resulting in a polynucleotide consisting partially of natural and partially of synthetic segments.

The polynucleotide either may code for the continuous stretch of the desired polypeptide or may code for two or more exons (each coding for a segment of the desired amino acid sequence) interrupted by one or more so-called introns (non-coding parts, which are excised during the process of expression of the polypeptide). Of course, such a polynucleotide containing exons can only be used successfully in eukaryotic cells.

The abovementioned polynucleotides according to the invention are transferred to a suitable prokaryotic or eukaryotic, advantageously a mammalian, host cell by first incorporating the polynucleotide into a vector and then transforming the host with this recombinant vector. In this transformed host the polynucleotide is located under the control of other polynucleotide sequences which regulate expression and where appropriate secretion of the polypeptide product.

The polynucleotide can be present in the host in an autonomously replicating vector, or can be stably integrated in the genetic material of the host.

It goes without saying that instead of the said modified AT III polypeptides use can be made also of smaller polypeptides containing the region of AT III essential for inhibition of factor IIa or factor Xa and containing the amino acid substitution(s) mentioned above. These polypeptides can be prepared by recombinant DNA technology or by conventional peptide synthesis.

Example

METHODS

RNA preparation

Total RNA was extracted from either human fetal or adult liver samples by the guanidinium-phenol method (Chirgwin et al, Biochemistry 18, 5294, 1979) followed by oligo (dT) cellulose chromatography for selection of poly A^+ mRNA (Maniatis et al., Cold Spring Harbor Laboratory "Molecular Cloning: A Laboratory Manual", 1982).

cDNA synthesis and cloning

Complementary DNA was prepared according to a published procedure (Gubler and Hoffman, Gene 25, 263, 1983), provided with Eco RI linkers (Pharmacia), and ligated into phage λgt10 (Promega). In vitro packaging, titration of recombinant phage in E. coli BNN102, and preparation of library DNA onto nitrocellulose filters was as described (Huynh et al., DNA Cloning Techniques "A Practical Approach", 1984; Maniatis et al., ibid.).

Clone identification

Oligodeoxynucleotides were synthesized using the phosphoramidite method on an Applied Biosystems 381A DNA synthesizer. A complementary probe was synthesized comprising a conserved region within human serine proteinase inhibitors (e.g. serpin antithrombin III: position 1358-1382 in figure 1). Low stringency conditions for hybridization and washing were as described (Ragg, Nucl. Acids Res. 14, 1073, 1986). A partial cDNA clone was then used as nick-translated probe (Maniatis et al., ibid.) to obtain full-length cDNAs.

Site directed mutagenesis

Oligonucleotide directed mutagenesis was performed using a cDNA fragment cloned in M13mp18/19 as a template essentially as described (Nisbet and Beilharz, Gene Anal. Techn. 2, 23, 1985). The uracil containing phage M13mp18/19 templates were prepared by their growth on E. coli RZ1032 (dut^-, ung^-) as mentioned (Kunkel, Proc.Natl.Acad.Sci. USA 82, 488, 1985). Mutants were screened by determining their nucleic acid sequence (Sanger et al., Proc. Natl. Acad. Sci. USA 74, 5463, 1977).

Construction of expression plasmid

Wild type and variant antithrombin III genes were inserted in the unique restriction endonuclease BamHI site of the vector pKCR (O'Hara et al., Proc. Natl. Acad. Sci. USA, 78, 1527, 1981) in which the last exon region of the rabbit β-globin gene was removed by digestion with restriction endonuclease EcoRI and BglII, filling in, and religation (position 1122-1196; Van Ooyen et al., Science 206, 337, 1979) and replacement of pBR322 for pBR327 sequences.

Mammalian cell expression and affinity purification

For transient expression recombinant constructs were introduced by the DEAE-chloroquine method (Luthman and Magnusson, Nucl. Acids Res. 5, 1295, 1983) in COS cells (Gluzman, Cell 23, 175, 1982).

Serum-free culture supernatants derived 24-72 h posttransfection were concentrated by Amicon YM10 filtration and dialyzed against 0.05 mol/l Tris-HCl pH 8.0, 0.1 mol/l NaCl.

For stable transformation recombinant constructs and pSV2neo (Colbère-Garapin et al., J.Mol.Biol. 150, 1, 1981) were introduced by the calcium phosphate precipitation method (Graham and van der Eb, Virology 52, 456, 1973) in CHO cells. To select CHO transfectants for the stable integration and expression of the neo gene, the antibiotic G418 (Gibco) was added 24 hours post-transfection at a concentration of 1 mg/ml. After 14 days, production of ATIII by pools of G418-resistant colonies was monitored by an ATIII-specific ELISA using goat anti-human ATIII IgG (Sera Lab), rabbit anti-human ATIII IgG (Dakopatts), and sheep anti-rabbit IgG HRP conjugate. 2.5 x 10^6 Cells were seeded and grown for 48 hours in medium containing 10% foetal calf serum. After washing serum-free culture supernatants were harvested after an additional 48 hours and concentrated/dialyzed as mentioned above. Low-affinity monoclonal antibody AATR-1 (Interferon Sciences) coupled to tresyl-activated Sepharose (Pharmacia) was added to the processed supernatant and incubated for 4 hours at room temperature. Elution of bound ATIII was performed for 10 minutes in a volume of 1 ml of 3 mol/l NaSCN, 0.01 mol/l Tris-HCl pH 8.0. Immediately after elution, samples were dialyzed against 0.05 mol/l Tris-HCl pH 8.0, 0.1 mol/l NaCl. The concentration of ATIII was determined by the above-mentioned ELISA.

Inhibition assays

The heparin-dependent factor X_a and factor II_a inactivating potency of antithrombin III variants has been investigated with the help of the continuously monitored enzyme inactivation assay (Tian and Tsou, Biochemistry 21, 1028, 1982). They are expressed as the percentage of inhibition relative to the wild type ATIII.

RESULTS

- A recombinant phage containing part of the antithrombin III structural gene was obtained from a human fetal liver cDNA library using the conserved serpin probe. Its ATIII origin was confirmed by positive hybridization with a reactive site oligonucleotide probe (position 1253-1294 in figure 1) as well as DNA sequence analysis. The partial cDNA ATIIIcDNA1 extends from position 887 to 1472. Two nucleotide differences were found when ATIIIcDNA1 was compared to the ATIII cDNA reported by Bock et al. (Nucl. Acids Res. 10, 8113, 1982); C at position 1049 and T at position 1317 both differences are silent mutations not changing the corresponding amino acid codons. The insert of ATIIIcDNA1 was used as a probe in subsequent screening of a human adult liver cDNA library resulting in ATIIIcDNA2 extending from position 75 to 1479 (Bock et al., ibid.). The incomplete ATIII leader sequence was replaced by a synthetic DNA leader derived from the published ATIII cDNA sequence (Bock et al., ibid.). In addition, upstream of the ATIII leader sequence a suitable restriction endonuclease EcoRI site was positioned at position -10 (5'-GAATTCCACCATG-3'). The complete ATIII cDNA cassette therefore consists of the following regions: i EcoRI (position -10) towards SacII (position 130) derived from synthetic origin, ii SacII (position 130) towards NcoI (position 935) derived from ATIIIcDNA2, and iii NcoI (position 935) towards EcoRI

(position 1474) derived from ATIIIcDNA1. All fragments were ligated in the unique restriction endonuclease EcoRI site of pBR327.

- Inspection of the ATIII cDNA sequence around the reactive centre (P-region) indicate the existence of surrounding restriction endonuclease PvuII (position 1241) and StuI (position 1312) sites. Since an additional restriction endonuclease PvuII site is present at position 1096 this was deleted by site directed mutagenesis of residue G_{1101} into T_{1101}, disturbing the restriction endonuclease PvuII recognition site, but still leaving the amino acid codon intact (CTG → CTT: Leu). Mutations introduced in the reactive centre P-region of ATIII can be performed by the replacement of the internal PvuII - StuI fragment (position 1241 to 1312) by a synthetic P-region of choice.

- Mutations introduced into the ATIII reactive centre P-region are derived from the human prothrombin gene (Degen and Davie, Biochemistry 26, 6165, 1987). Prothrombin participates in the final stage of clot formation since it is activated to thrombin by factor X_a. Selection of prothrombin sequences known to be physiological substrates for factor X_a and integration of (parts of) these sequences within the ATIII reactive centre P-region influences the inhibition profile of ATIII between factor X_a and factor II_a. The different synthetic P-regions, being hybrids between ATIII and prothrombin sequences, are illustrated in figure 2; they were used to replace the wild type PvuII - StuI fragment of the original ATIII cDNA backbone in pBR327 and confirmed by DNA sequence analysis.

- ATIII variants are evaluated for their inhibition profile in the following way. The variant ATIII containing fragments are isolated by digestion with restriction endonuclease EcoRI and filling in by DNA

polymerase. The blunt-ended fragments are then ligated in pKCR digested with restriction endonuclease BamHI and filling in by DNA polymerase. Correct orientation of the fragments with respect to the SV40 promoter was checked by restriction endonuclease mapping. The resulting recombinants were used for transient and stable expression in COS and CHO cells, respectively. Culture supernatants were tested for in vitro heparin-dependent inhibitory activity towards factor Xa and factor IIa.
- The ATIII P-region variants possessing different amino acid substituents derived from the prothrombin sequence (i.e. factor Xa cleavage site related to the formation of meizothrombin) are summarized in figure 2. As shown in figures 3 and 4 substitutions introduced in this way result in a remarkable shift in the heparin-dependent inhibition profile of ATIII towards factors IIa and Xa. With respect to the ATIII variant class being specific for factor Xa this can be accomplished by two independent ways. Either modification of at least amino acid residue 394 (Ser^{394} → Ile) or modification of amino acid residues 391 and 396 (Ala^{391} → Asp and Asn^{396} → Glu) result in a common inhibition profile of the modified ATIII. In addition, the nature of the amino acid substitution at position 394 is crucial in conferring inhibitory profile to the ATIII polypeptide (figures 5 and 6). Therefor, other amino acid substitutions at position 394 that represent a large and/or hydrofilic character (Chotia, Ann.Rev.Biochem. 33, 537, 1984) predict a specificity of heparin-dependent inhibition towards factor Xa.
Finally, modelling studies of factor Xa support the requirement of negatively charged residues within ATIII at the P3 and P3' position in order to form salt bridges with positively charged residues present in the S3 and S3' binding sites (Greer, J.Mol.Biol. 153, 1043, 1981).

Figure 1:

Antithrombin III cDNA sequence of reconstructed cassette.

Asterisks indicate differences observed with the ATIII cDNA sequence of Bock et al. (ibid.). Broken underlined restriction endonuclease sites were used for reconstruction experiments.

Underlined restriction endonuclease sites were used for P region replacement studies. The nucleotide G_{1101} indicted by 0 was mutagenized towards T_{1101} for deletion of the additional restriction endonuclease PvuII site.

The arrows indicate the start positions of partial ATIII cDNA clones obtained during this study.

Figure 2:

Antithrombin III P-region variants (PvuII-StuI: position 1244-1314)
Antithrombin III (ATIII; Bock et al., ibid)
Prothrombin (FII; Degen and Davie, ibid)
Amino acids that differ from the natural ATIII polypeptide are underlined.

Figure 3:

Heparin-dependent inhibition of factors IIa and Xa of ATIII and variants of culture supernatants derived from transfected COS cells.
WT = ATIII, COS = COS control and T = Tris-buffer.

Figure 4/Figure 6:

Heparin-dependent inhibition of factors IIa and Xa of ATIII and variants after affinity purification of culture supernatants derived from transfected CHO cells. WT = ATIII, C = CHO control, CT = transfected CHO control and T = Tris buffer.

Figure 5:

Antithrombin III P_1' variants

Amino acids that differ from the natural ATIII polypeptide are underlined.

Claims

1. Modified antithrombin III polypeptide, characterized in that it differs from natural mature antithrombin III protein in that at least one amino acid from the region comprising amino acids 384-396 is replaced by the corresponding amino acid(s) grouped around the factor Xa cleavage site in factor II related to the formation of meizothrombin, or a fragment thereof.

2. Modified antithrombin III polypeptide or a fragment thereof according to claim 1, wherein at least one of the following amino acid substitutions has taken place:
$Ala^{384} \rightarrow Glu$
$Ser^{385} \rightarrow Leu$
$Thr^{386} \rightarrow Leu$
$Ala^{387} \rightarrow Glu$
$Val^{388} \rightarrow Ser$
$Val^{389} \rightarrow Tyr$
$Ala^{391} \rightarrow Asp$
$Ser^{394} \rightarrow Ile.$
$Asn^{396} \rightarrow Glu$

3. Modified antithrombin III polypeptide or a fragment thereof according to claim 1 or 2, wherein at least the following amino acid substitution has taken place:
$Ser^{394} \rightarrow Ile.$

4. Modified antithrombin III polypeptide or a fragment thereof according to claim 1 or 2, wherein at least the following amino acid substitution has taken place:
$Ala^{391} \rightarrow Asp.$

5. Modified antithrombin III polypeptide or a fragment thereof according to claim 1 or 2, wherein at least the following amino acid substitution has taken place:
$Asn^{396} \rightarrow Glu.$

6. Modified antithrombin III polypeptide or a fragment thereof according to claim 4 or 5, wherein at least the following amino acid substitutions have taken place: $Ala^{391} \rightarrow Asp$ and $Asn^{396} \rightarrow Glu$.

7. Nucleic acid sequence at least part of which is coding for a modified antithrombin III polypeptide or a fragment thereof according to claim 1-6.

8. Nucleic acid sequence according to claim 7, furthermore comprising at the 5' terminus of the sequence coding for the polypeptide a signal sequence and/or a promoter.

9. Vector comprising a nucleic acid sequence according to claim 7-8.

10. Host cell containing a nucleic acid sequence according to claim 7-8 or a vector according to claim 9.

11. Host cell according to claim 10 characterized in that it is derived from a mammalian cell.

12. Pharmaceutical preparation containing a polypeptide or a fragment thereof according to claim 1-6 as well as a pharmaceutically acceptable carrier.

FIGURE 1

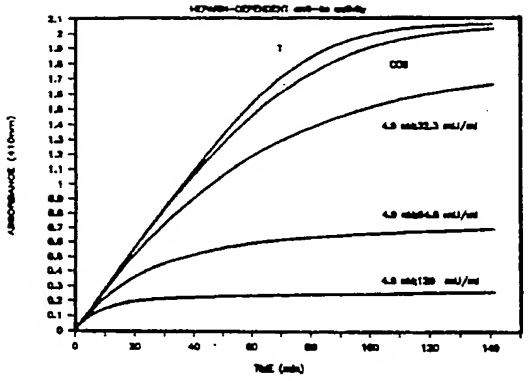
```
                                                        5'-GAATT CCACC
    0 ATGTA TTCCA ATGTG ATAGG AACTG TAACC TCTGG AAAAA GGAAG GTTTA
   50 TCTTT TGTCC TTGCT GCTCA TTGGC TTCTG GGACT GCGTG ACCTG TCACG
  100 GGAGC CCTGT GGACA TCTGC ACAGC CAAGC CGCGG GACAT TCCCA TGAAT
  150 CCCAT GTGCA TTTAC CGCTC CCCGG AGAAG AAGGC AACTG AGGAT GAGGG
  200 CTCAG AACAG AAGAT CCCGG AGGCC ACCAA CCGGC GTGTC TGGGA ACTGT

  250 CCAAG GCCAA TTCCC GCTTT GCTAC CACTT TCTAT CAGCA CCTGG CAGAT
  300 TCCAA GAATG ACAAT GATAA CATTT TCCTG TCACC CCTGA GTATC TCCAC
  350 GGCTT TTGCT ATGAC CAAGC TGGGT GCCTG TAATG ACACC CTCCA GCAAC
  400 TGATG GAGGT ATTTA AGTTT GACAC CATAT CTGAG AAAAC ATCTG ATCAG
  450 ATCCA CTTCT TCTTT GCCAA ACTGA ACTGC CGACT CTATC GAAAA GCCAA

  500 CAAAT CCTCC AAGTT AGTAT CAGCC AATCG CCTTT TTGGA GACAA ATCCC
  550 TTACC TTCAA TGAGA CCTAC CAGGA CATCA GTGAG TTGGT ATATG GAGCC
  600 AAGCT CCAGC CCCTG GACTT CAAGG AAAAT GCAGA GCAAT CCAGA GCGGC
  650 CATCA ACAAA TGGGT GTCCA ATAAG ACCGA AGGCC GAATC ACCGA TGTCA
  700 TTCCC TCGGA AGCCA TCAAT GAGCT CACTG TTCTG GTGCT GGTTA ACACC

  750 ATTTA CTTCA AGGGC CTGTG GAAGT CAAAG TTCAG CCCTG AGAAC ACAAG
  800 GAAGG AACTG TTCTA CAAGG CTGAT GGAGA GTCGT GTTCA GCATC TATGA
  850 TGTAC CAGGA AGGCA AGTTC CGTTA TCGGC GCGTG GCTGA AGGCA CCCAG
  900 GTGCT TGAGT TGCCC TTCAA AGGTG ATGAC ATCAC CATGG TCCTC ATCTT
  950 GCCCA AGCCT GAGAA GAGCC TGGCC AAGGT GGAGA AGGAA CTCAC CCCAG

1,000 AGGTG CTGCA GGAGT GGCTG GATGA ATTGG AGGAG ATGAT GCTGG TGGTC
1,050 CACAT GCCCC GCTTC CGCAT TGAGG ACGGC TTCAG TTTGA AGGAG CAGCT
1,100 GCAAG ACATG GGCCT TGTCG ATCTG TTCAG CCCTG AAAAG TCCAA ACTCC
1,150 CAGGT ATTGT TGCAG AAGGC CGAGA TGACC TCTAT GTCTC AGATG CATTC
1,200 CATAA GGCAT TTCTT GAGGT AAATG AAGAA GGCAG TGAAG CAGCT GCAAG

1,250 TACCG CTGTT GTGAT TGCTG GCCGT TCGCT AAACC CCAAC AGGGT GACTT
1,300 TCAAG GCCAA CAGGC CTTTC CTGGT TTTTA TAAGA GAAGT TCCTC TGAAC
1,350 ACTAT TATCT TCATG GGCAG AGTAG CCAAC CCTTG TGTTA AGTAA AATGT
1,400 TCTTA TTCTT TGCAC CTCTT CCTAT TTTTG GTTTG TGAAC AGAAG TAAAA
1,450 ATAAA TACAA ACTAC TTCCA TCGGA ATTC - 3'
```

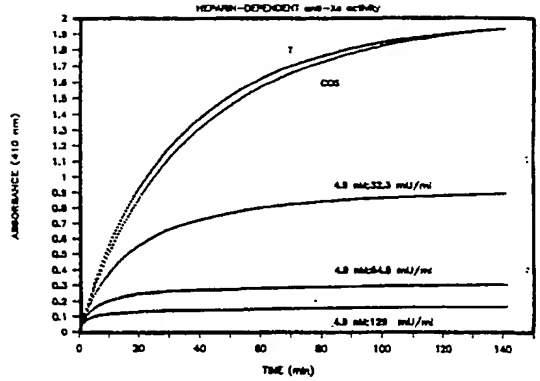
Figure 2

	385						390			P_1	P_1'	395					400				
ATIII	Ala	Ala	Ser	Thr	Ala	Val	Val	Ile	Ala	Gly	Arg	Ser	Leu	Asn	Pro	Asn	Arg	Val	Thr	Phe	Lys	Ala
FII	Arg	Glu	Leu	Leu	Glu	Ser	Tyr	Ile	Asp	Gly	Arg	Ile	Val	Glu	Gly	Ser	Asp	Ala	Glu	Ile	Gly	Met
Mutant 1	Ala	Glu	Leu	Leu	Glu	Ser	Tyr	Ile	Asp	Gly	Arg	Ile	Val	Glu	Gly	Ser	Asp	Ala	Glu	Ile	Gly	Ala
Mutant 2	Ala	Glu	Leu	Leu	Glu	Ser	Tyr	Ile	Asp	Gly	Arg	Ser	Leu	Asn	Pro	Asn	Arg	Val	Thr	Phe	Lys	Ala
Mutant 3	Ala	Ala	Ser	Thr	Ala	Val	Val	Ile	Asp	Gly	Arg	Ile	Val	Glu	Pro	Asn	Arg	Val	Thr	Phe	Lys	Ala
Mutant 4	Ala	Ala	Ser	Thr	Ala	Val	Val	Ile	Asp	Gly	Arg	Ile	Leu	Glu	Pro	Asn	Arg	Val	Thr	Phe	Lys	Ala
Mutant 5	Ala	Ala	Ser	Thr	Ala	Val	Val	Ile	Asp	Gly	Arg	Ile	Leu	Asn	Pro	Asn	Arg	Val	Thr	Phe	Lys	Ala
Mutant 6	Ala	Ala	Ser	Thr	Ala	Val	Val	Ile	Ala	Gly	Arg	Ile	Leu	Glu	Pro	Asn	Arg	Val	Thr	Phe	Lys	Ala
Mutant 7	Ala	Ala	Ser	Thr	Ala	Val	Val	Ile	Asp	Gly	Arg	Ser	Leu	Glu	Pro	Asn	Arg	Val	Thr	Phe	Lys	Ala
Mutant 8	Ala	Ala	Ser	Thr	Ala	Val	Val	Ile	Asp	Gly	Arg	Ser	Leu	Asn	Pro	Asn	Arg	Val	Thr	Phe	Lys	Ala
Mutant 9	Ala	Ala	Ser	Thr	Ala	Val	Val	Ile	Ala	Gly	Arg	Ser	Leu	Glu	Pro	Asn	Arg	Val	Thr	Phe	Lys	Ala
Mutant 10	Ala	Ala	Ser	Thr	Ala	Val	Val	Ile	Ala	Gly	Arg	Ile	Leu	Asn	Pro	Asn	Arg	Val	Thr	Phe	Lys	Ala

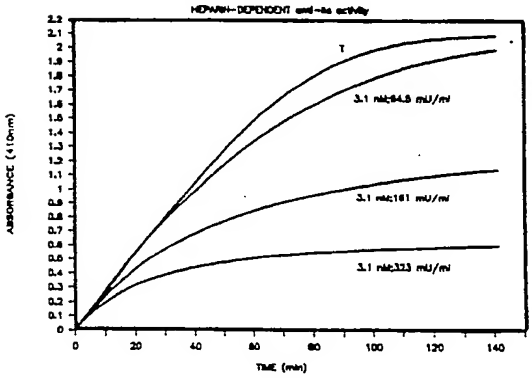
Figure 3

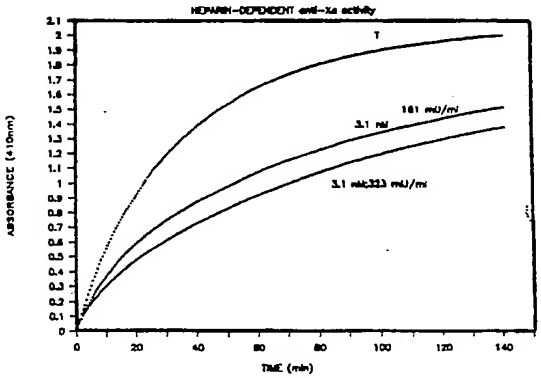
Antithrombin III wild type (WT)



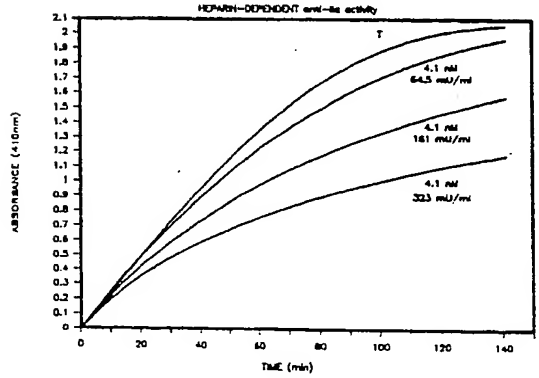


Antithrombin III mutant 2





Antithrombin III mutant 3



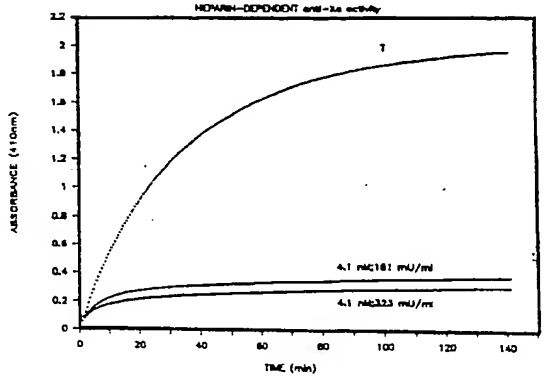


Figure 4

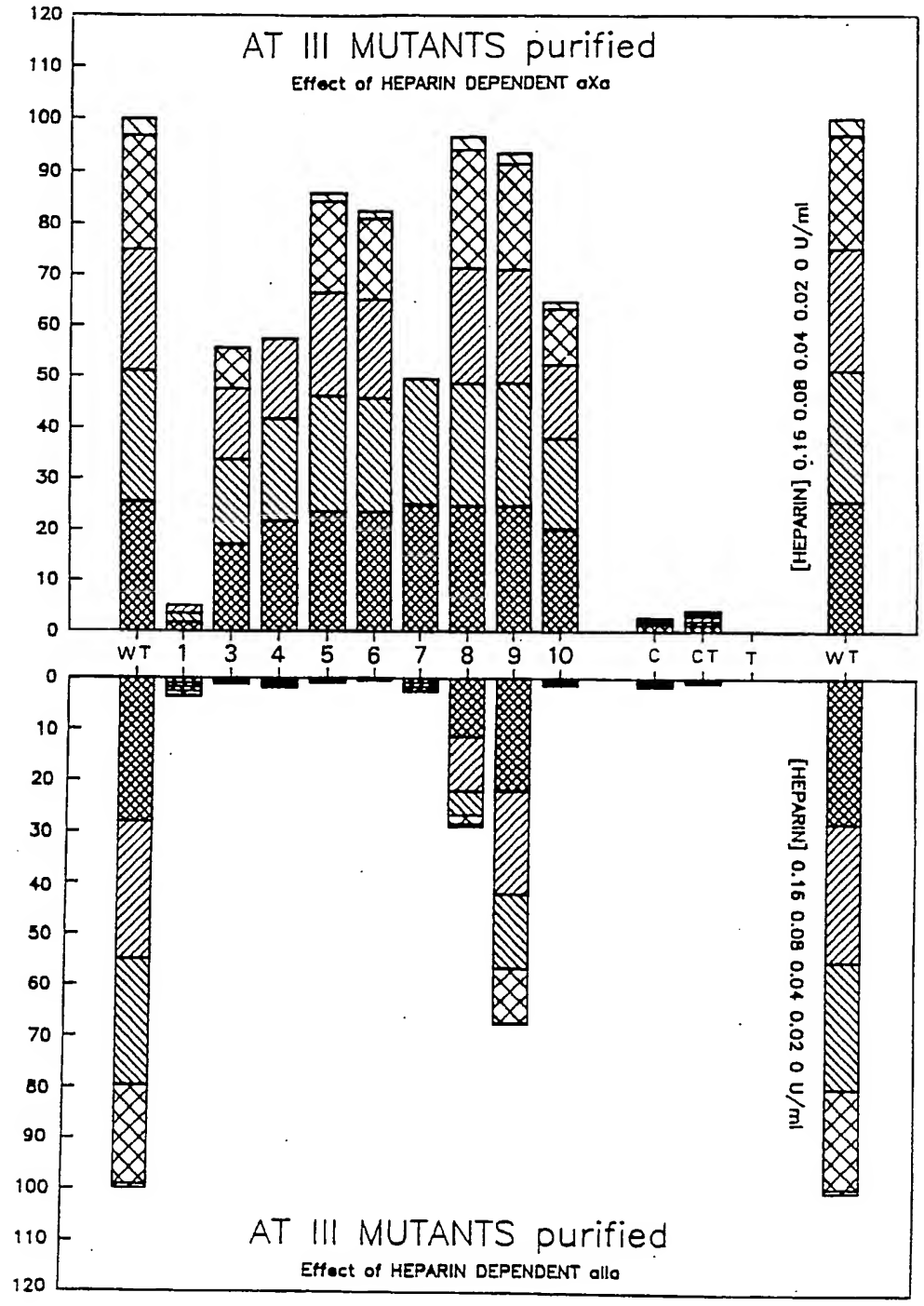
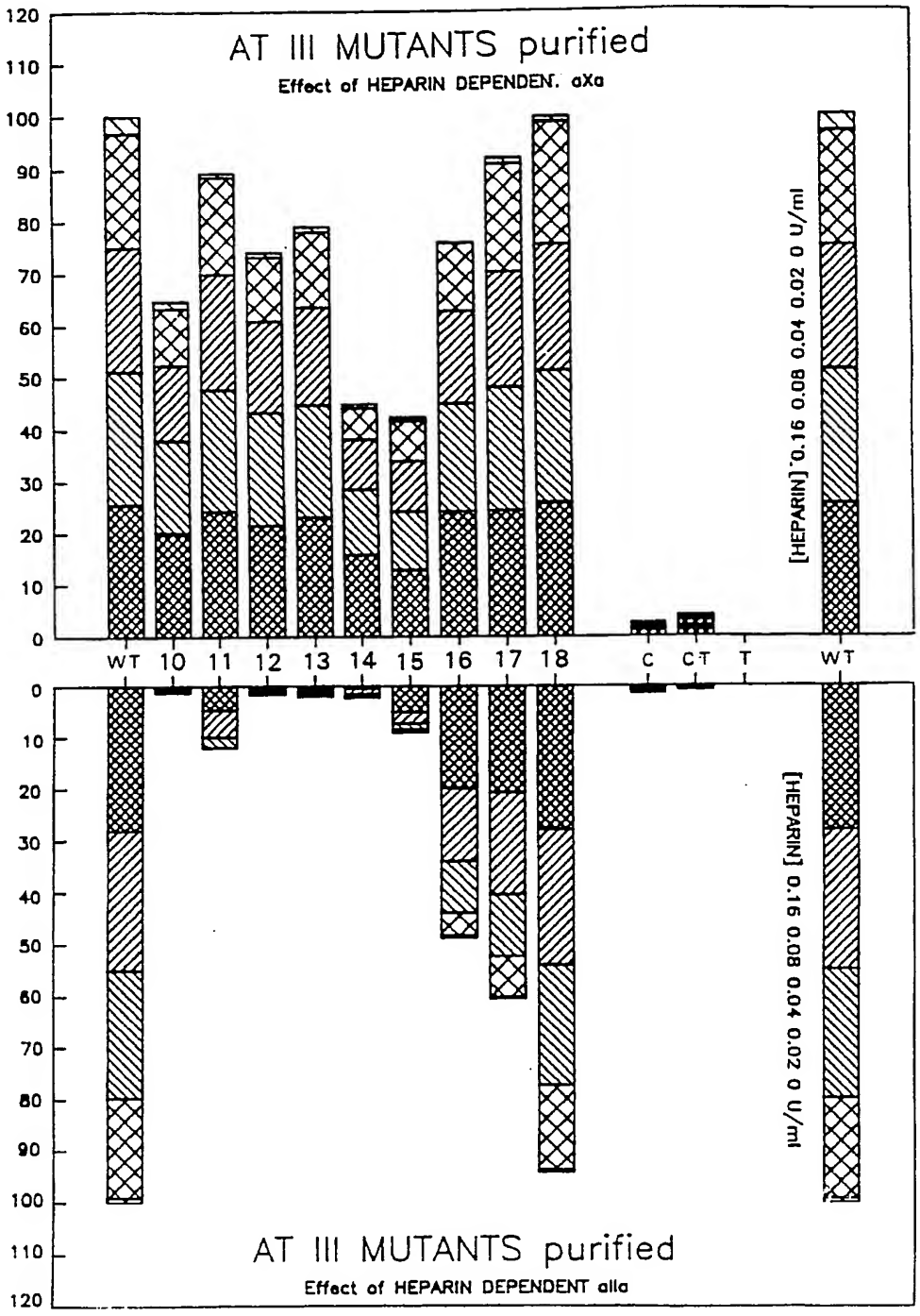

AT III MUTANTS purified
Effect of HEPARIN DEPENDENT aXa
[HEPARIN] 0.16 0.08 0.04 0.02 0 U/ml
WT 1 3 4 5 6 7 8 9 10 C CT T WT
AT III MUTANTS purified
Effect of HEPARIN DEPENDENT alla
[HEPARIN] 0.16 0.08 0.04 0.02 0 U/ml

Figure 5

	390				P_1	P_1'	395			
ATIII	Val	Ile	Ala	Gly	Arg	Ser	Leu	Asn	Pro	Asn
Mutant 10	Val	Ile	Ala	Gly	Arg	Ile	Leu	Asn	Pro	Asn
Mutant 11	Val	Ile	Ala	Gly	Arg	Phe	Leu	Asn	Pro	Asn
Mutant 12	Val	Ile	Ala	Gly	Arg	Tyr	Leu	Asn	Pro	Asn
Mutant 13	Val	Ile	Ala	Gly	Arg	Leu	Leu	Asn	Pro	Asn
Mutant 14	Val	Ile	Ala	Gly	Arg	Val	Leu	Asn	Pro	Asn
Mutant 15	Val	Ile	Ala	Gly	Arg	Met	Leu	Asn	Pro	Asn
Mutant 16	Val	Ile	Ala	Gly	Arg	Thr	Leu	Asn	Pro	Asn
Mutant 17	Val	Ile	Ala	Gly	Arg	Gly	Leu	Asn	Pro	Asn
Mutant 18	Val	Ile	Ala	Gly	Arg	Ala	Leu	Asn	Pro	Asn

Figure 6


AT III MUTANTS purified
Effect of HEPARIN DEPENDEN. aXa
[HEPARIN] 0.16 0.08 0.04 0.02 0 U/ml
WT 10 11 12 13 14 15 16 17 18 C CT T WT
[HEPARIN] 0.16 0.08 0.04 0.02 0 U/ml
AT III MUTANTS purified
Effect of HEPARIN DEPENDENT alla

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 90/01026

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 07 K 13/00, C 12 N 15/15, 15/85, 5/10, A 61 K 37/64

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 07 K, C 12 N, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	The Journal of Biological Chemistry, volume 263, no. 31, 5 November 1988, The American Society For Biochemistry and Molecular Biology, Inc., (US), A.W. Stephens et al.: "Site-directed mutagenesis of the reactive center (serine 394) of antithrombin III", pages 15849-15852 see the whole article	1-12
A	Proc. Natl. Acad. Sci. USA, volume 84, no. 11, June 1987, (Washington, D.C., US), A.W. Stephens et al.: "Expression of functionally active human anti-thrombin III", pages 3886-3890 see the whole article	7-11
A	EP, A, 0238473 (MONSANTO) 23 September 1987 see page 14, peptide 18; page 17, peptide 18; page 20, peptide 18	1-12

* Special categories of cited documents: [10]

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
8th October 1990	31 OCT 1990
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme N. KUIPER

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9001026 SA 38201

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 26/10/90
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0238473	23-09-87	JP-A- 62277399	02-12-87

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